THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 9, pp. 7123–7131, March 4, 2011

Mutation to Bax beyond the BH3 Domain Disrupts Interactions with Pro-survival Proteins and Promotes Apoptosis*[S]

Received for publication, July 5, 2010, and in revised form, December 15, 2010 Published, JBC Papers in Press, January 3, 2011, DOI 10.1074/jbc.M110.161281

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Pro-survival members of the Bcl-2 family of proteins restrain the pro-apoptotic activity of Bax, either directly through interactions with Bax or indirectly by sequestration of activator BH3-only proteins, or both. Mutations in Bax that promote apoptosis can provide insight into how Bax is regulated. Here, we describe crystal structures of the pro-survival proteins Mcl-1 and Bcl-x_L in complex with a 34-mer peptide from Bax that encompasses its BH3 domain. These structures reveal canonical interactions between four signature hydrophobic amino acids from the BaxBH3 domain and the BH3-binding groove of the pro-survival proteins. In both structures, Met-74 from the Bax peptide engages with the BH3-binding groove in a fifth hydrophobic interaction. Various Bax Met-74 mutants disrupt interactions between Bax and all pro-survival proteins, but these Bax mutants retain pro-apoptotic activity. Bax/Bak-deficient mouse embryonic fibroblast cells reconstituted with several Bax Met-74 mutants are more sensitive to the BH3 mimetic compound ABT-737 as compared with cells expressing wild-type Bax. Furthermore, the cells expressing Bax Met-74 mutants are less viable in colony assays even in the absence of an external apoptotic stimulus. These results support a model in which direct restraint of Bax by pro-survival Bcl-2 proteins is a barrier to apoptosis.

The Bcl-2 family of proteins plays a central role in the control of apoptosis via the intrinsic pathway (1). The family is composed of two opposing groups, one promoting cell survival (comprising Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1, each containing up to four Bcl-2 homology (BH)[3] domains) and another promoting cell death. This latter group includes Bax and Bak, proteins similar in sequence and structure to the pro-survival family members, and the so-called BH3-only proteins. In response to stress or damage signals, BH3-only proteins are up-regulated either transcriptionally or post-translationally, or both. Apoptosis results when the pro-survival proteins are overwhelmed and Bax/Bak undergo a conformational change at the mitochondrial membrane. Apoptogenic factors such as cytochrome *c* are then released from the mitochondrial intramembrane space into the cytosol, resulting in the activation of caspases.

Many biochemical, structural, and cellular studies support an interaction between pro-survival proteins and both subgroups of pro-apoptotic proteins. The helical BH3 domain of pro-apoptotic proteins engages a cognate groove on the pro-survival proteins (2–5). Four hydrophobic amino acids from the BH3 domain insert into pockets in the groove, and a conserved aspartyl residue in the BH3 domain is hydrogen-bonded with a conserved arginyl residue on the pro-survival protein. Although the BH3-only proteins are mostly unstructured in solution (6), Bak and Bax have well characterized globular structures (7, 8), remarkably similar to the pro-survival proteins, in which the BH3 domain is located within an amphipathic helix (α2) with its hydrophobic face buried. Thus Bak and Bax cannot bind to pro-survival proteins without first everting their BH3 domains. The natural trigger for the exposure of the BH3 domain of Bak/Bax is not known, but it could be a chemical stimulus such as H_2O_2 (9), a physical stimulus such as heat (10), or an interaction between Bak/Bax and a BH3-only protein that is up-regulated in response to cellular stresses. Some evidence supports such interactions, at least between a subset of the BH3-only proteins and Bax/Bak (11–15). The isolation of complexes of this type has proven elusive, but a recent NMR study suggests the BH3 domain of the BH3-only protein Bim binds Bax on the opposite face as compared with where a BH3 peptide binds a pro-survival protein (16). It has been proposed that this interaction triggers a structural rearrangement in Bax or Bak into large homo-oligomeric complexes at the mitochondrial membrane. Other data support a model in

* This work was supported by a fellowship from the Australian Research Council (to P. E. C.), National Health and Medical Research Council (NHMRC) Australia Project Grant 575561 (to P. E. C.), Program Grant 461221 fellowships (to W. D. F. and P. M. C.), Leukemia and Lymphoma Society (SCOR 7015-02), Cancer Council of Victoria Grant-in-Aid 461239 (to W. D. F.), a Leukaemia Foundation of Australia Phillip Desbrow Postdoctoral Fellowship (to E. F. L.), and an Australian Cancer Research Foundation grant (to P. M. C.).

The atomic coordinates and structure factors (codes 3PK1 and 3PL7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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[3] The abbreviations used are: BH, Bcl-2 homology; MEF, mouse embryonic fibroblast; IRES, internal ribosomal entry site; DMSO, dimethyl sulfoxide.

which Bak and Bax are free to oligomerize only after the constraint of binding to a pro-survival protein has been relieved (17–20). Most recently, the Andrews laboratory (21–23) has provided evidence for an "embedded together" model, incorporating elements of both the direct and the indirect activation models of Bax and Bak.

Previous studies of Bax mutants have led to diverse conclusions. It has been reported that the quadruple mutant (L63A/G67A/L70A/M74A) can neither homodimerize nor heterodimerize with Bcl-2 or Bcl-x_L, yet it induces apoptosis when overexpressed in Jurkat cells (24). The double mutant L70A/D71A is reported to no longer bind to Bcl-2, Bcl-x_L, or Mcl-1 and to be partially active in cell killing when reconstituted into Bax/Bak doubly deficient cells (25). No cell killing was reported for this Bax mutant, nor the equivalent Bak mutant (I82A/N83A), without an apoptotic stimulus such as treatment with etoposide (25). Another study using GFP-Bax constructs shows that Bax L70A and Bax D71A are both localized to mitochondria and constitutively active (26). A further study shows that Bax D68R co-immunoprecipitates with none of the pro-survival family members and kills unstimulated cells, but only if Bcl-x_L is absent or down-regulated (17). The double mutant Bax D68R/S184L is predominantly associated with the mitochondrial membrane and is constitutively active without the requirement to delete Bcl-x_L (17).

Here, we report structures of the pro-survival proteins Mcl-1 and Bcl-x_L in complex with a peptide that encompasses the BaxBH3 domain plus flanking residues. These structures resemble previous structures of pro-survival proteins in complex with other BH3 domains, with interactions dominated by 4 conserved hydrophobic residues and a conserved salt bridge. In addition, we observe that Met-74, outside the signature BH3 domain, interacts with the hydrophobic groove of the pro-survival protein. Mutagenesis of Met-74 disrupts interaction between Bax and full-length pro-survival proteins in cells, confirming its role in these interactions. The Met-74 mutants do not compromise the killing activity of Bax. On the contrary, cells expressing the mutants have reduced viability as compared with those expressing wild-type Bax. These data provide further evidence that pro-survival proteins inhibit apoptosis through directly engaging Bax.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Human Mcl-1 with an N-terminal truncation (ΔN173) and C-terminal truncation (ΔC27) was cloned between the BamHI and EcoRI sites of pET32a (Novagen Inc.). The resultant expressed protein encoded the Bcl-2-like region of Mcl-1, thus lacking the N-terminal PEST domain in addition to the C-terminal trans-membrane domain. The protein was expressed in *Escherichia coli* strain BL21 (DE3) as a thioredoxin-His tag fusion, purified on Ni^{2+}-nitrilotriacetic acid-agarose (Bio-Rad), thrombin-digested, and further purified by size exclusion on a Superdex 75 16/60 column (GE Healthcare) in 20 mM Tris, pH 8.0, 150 mM NaCl. Human Bcl-x_L containing a shortened $\alpha1-\alpha2$ loop (Δaa 45–84) and C-terminal truncation (ΔC25) (27) was expressed and purified as described previously (28). Bax peptide encompassing the BH3 domain and adjacent residues was purchased as a synthetic 34-mer peptide from Mimotopes (Victoria, Australia; sequence = DPVPQDASTKKLSECLKRIGDELDSNMELQRMIA).

Crystallization, Data Collection, and Processing—Mcl-1 was mixed with an equimolar amount of BaxBH3 peptide and concentrated to 10 mg/ml. Crystals were grown by the hanging drop method at 295 K (reservoir solution; 1 M sodium acetate, 0.1 M Hepes, pH 7.0, 25 mM $CdSO_4$, 5 mM tris(2-carboxyethyl)-phosphine). Crystals were equilibrated in cryo-protectant consisting of reservoir solution supplemented with 25% (v/v) ethylene glycol and then flash-frozen in liquid N_2. X-ray data were collected at the Australian Synchrotron on beamline MX1 at 100 K. The data were processed using HKL2000 (29). The structure was solved by molecular replacement with PHASER (30) using Mcl-1 from the published Mcl-1·BimBH3 complex as a search model ((5); Protein Data Bank (PDB) 2NL9). Several rounds of building in COOT (31) and refinement in REFMAC5 (32) led to an early model with high *R*-factors. Further analysis of the data with XTRIAGE (33) identified twinning with twin law h + 2 × l, −k, and −l and a twin fraction of 0.27. Subsequent refinement was performed in PHENIX (34, 35) and incorporated simulated annealing, TLS, non-crystallographic symmetry, individual site refinement, individual ADP refinement, and detwinning. The final refinement statistics for the model are given in Table 1.

Bcl-x_L·BaxBH3 peptide complex was prepared and crystallized as described for Mcl-1·BaxBH3 peptide complex except that a reservoir solution of 13% (w/v) PEG 6000, 0.2 M $CaCl_2$, 0.1 M Tris, pH 8.0, was used. Crystals were equilibrated into reservoir supplemented with 20% (v/v) ethylene glycol and flash-frozen in liquid N2. X-ray data were collected at beamline MX1 at the Australian Synchrotron and processed with HKL2000 (29). The structure was solved by molecular replacement with PHASER (30) using Bcl-x_L Chain A coordinates from PDB file 2YXJ (28). Several rounds of building in COOT (31) and refinement in REFMAC5 (32) and PHENIX (34, 35) incorporating simulated annealing were performed to produce the final model. Structural figures were prepared with PyMOL (DeLano Scientific).

Biacore Analysis—Solution competition assays were performed using a Biacore 3000 instrument as described previously (36). Pro-survival proteins (5–10 nM) were incubated with increasing concentrations of wild-type or mutant BaxBH3 peptides for 2 h in running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Tween 20, pH 7.4) prior to injection onto a CM5 chip onto which either a wild-type 26-mer BimBH3 peptide or an inert BimBH3 mutant peptide (Bim4E) was immobilized. Specific binding of the pro-survival protein to the surface in the presence and absence of peptides was quantified by subtracting the signal from the Bim4E channel from that obtained on the wild-type Bim channel. The ability of the peptides to prevent protein binding to immobilized BimBH3 was expressed as the IC_{50}, calculated by nonlinear curve fitting of the data with KaleidaGraph (Synergy Software).

Yeast Colony Assays—Saccharomyces cerevisiae (strain W303α) growth inhibition assays were performed as described

previously (37). Yeast were transformed with plasmids encoding for pro-survival proteins (pGALL-(*Trp1*) vector) or Bax mutants with an N-terminal FLAG tag (pGALS-(*Leu2*) vector). Cells were grown overnight under selection in minimal medium lacking Trp and Leu. Cells were washed twice in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and then resuspended in TE buffer to yield a final concentration of 6250 colony-forming units/5 μl of suspension. Serial 5-fold dilutions were then spotted onto plates containing either galactose (inducing, "ON") or glucose ("OFF") as a carbon source. Plates were incubated for 48–72 h and then photographed.

Co-immunoprecipitation—For co-immunoprecipitation/Western blotting experiments, HA-tagged pro-survival proteins were co-transfected with FLAG-tagged wild-type Bax or Bax mutants into HEK293T cells using Lipofectamine™ (Invitrogen). Cell lysates were prepared in lysis buffer (20 mM Tris, pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol) containing 1% (v/v) Triton X-100 and supplemented with protease inhibitors (Roche Applied Science). The cell lysates were incubated with anti-FLAG M2 affinity resin (Sigma). The bound proteins were eluted by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE, and transferred onto nitrocellulose membranes. HA-tagged proteins associated with immunoprecipitated pro-survival proteins were detected with the anti-HA antibody (3F10, Roche Applied Science). FLAG-tagged proteins were detected with an anti-FLAG antibody (9H1, Lorraine O'Reilly, WEHI, Australia). Relative expression levels of HA- and FLAG-tagged proteins were assessed by Western blot analysis on equal volumes of whole cell lysates. Blots were probed with anti-FLAG and anti-HA antibodies as above.

Cell Killing—Retroviral expression constructs were made using the pMIG vector (Murine Stem Cell Virus-IRES-GFP; the GFP sequence is that of EGFP) as described previously. These plasmids were transiently transfected, using Lipofectamine™ (Invitrogen), into Phoenix ecotropic packaging cells. Filtered virus-containing supernatants were used to infect SV40 large T-antigen transformed $bax^{-/-}bak^{-/-}$ mouse embryonic fibroblasts by spin inoculation as described previously (19). Cells stably expressing Bax or Bax mutants were selected by sorting GFP^{+ve} cells 24 h after spin inoculation. These stably expressing cells, as well as $bak^{-/-}$ and $bax^{-/-}bak^{-/-}$ MEFs, were treated with the indicated concentrations of etoposide or an equivalent volume of solvent (DMSO) control, and cell viability was monitored by propidium iodide (5 μg/ml) exclusion. For long term survival (colony) assays, 150 GFP^{+ve} cells/well were plated in triplicate in 6-well plates, 24 h after spin inoculation. After 6 days, colonies were stained with Coomassie Blue and counted. Colony assays performed in combination with ABT-737 were done essentially as described above except that cells were incubated with the indicated concentrations of ABT-737, which was added immediately after the GFP^{+ve} cells were sorted. To monitor the percentage of cells expressing Bax Met-74 mutants, cells were analyzed using a FACScan instrument (BD Biosciences) for infected cells (*i.e.* GFP^{+ve}; FL-1) 10 days after the original GFP^{+ve} population had been sorted.

Cell Fractionation—Mouse embryonic fibroblasts ($bax^{-/-}bak^{-/-}$) stably expressing either wild-type Bax or Bax mutants ($\sim 2 \times 10^6$ cells) were permeabilized by incubation in 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.05% (w/v) digitonin (Calbiochem) (supplemented with protease inhibitors (Roche Applied Science)) for 30 min at 30 °C before pelleting. The supernatant was retained as the soluble fraction, whereas the pellet, which contains intact mitochondria, was solubilized in 1% (v/v) Triton X-100-containing lysis buffer (20 mM Tris, pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol) supplemented with protease inhibitors (Roche Applied Science). Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The cytochrome *c* and Bax proteins were detected with the anti-cytochrome *c* (7H8.2C12, BD Pharmingen) and anti-FLAG (9H1, Lorraine O'Reilly) antibodies, respectively.

RESULTS

Crystal Structure of the Mcl-1·BaxBH3 Complex—Mcl-1 (ΔN173, ΔC27) was crystallized with a peptide encompassing the canonical 12-residue BaxBH3 domain, as delineated by the 4 conserved hydrophobic residues (residues 57–72) with 11 residues both N-terminal and C-terminal to this domain, the full peptide being Bax residues 48–81 (Fig. 1*A*). The crystals contain two complexes of Mcl-1·BaxBH3 within the asymmetric unit (root mean square deviation of Mcl-1 molecules (Chain A *versus* Chain C) = 0.06 Å, root mean square deviation of BaxBH3 domains (Chain B *versus* Chain D) = 0.03 Å). Data collection and refinement statistics are detailed in Table 1. The structure of the Mcl-1·BaxBH3 complex adopts a similar overall fold to previously described complexes of Mcl-1 with BH3 domains from BH3-only proteins (5, 38). In particular, Bax Asp-68 is hydrogen-bonded with Mcl-1 Arg-263, and Bax Leu-59, Leu-63, Ile-66, and Leu-70 project into the four canonical hydrophobic pockets within the peptide-binding groove of Mcl-1. In addition, Bax Met-74 functions as a 5th hydrophobic residue along the BH3 peptide, not unlike Bim Tyr-73 in the Mcl-1·BimBH3 complex (5). Of note, the secondary structure of the observed region of the Bax peptide within the complex (residues 54–80) is entirely helical, unlike the equivalent region in the structure of inactive full-length Bax (8) (Fig. 1).

Crystal Structure of the Bcl-x_L·BaxBH3 Complex—Bcl-x_L (Δaa 45–84, ΔC25) (27) was crystallized with the same peptide described above for the Mcl-1·BaxBH3 complex. The structure is unusual in that the asymmetric unit contains one copy of the BaxBH3 sandwiched between two copies of Bcl-x_L. One interface (Fig. 1*B*, Bcl-x_L Chain A to BaxBH3) represents the canonical Bcl-x_L BH3 interface and is similar to that observed for the Mcl-1·BaxBH3 complex described above and other previously described complexes of Bcl-x_L with other BH3 domains (2–4, 39). The second interface has not previously been observed and involves interactions between Asp-71 (mimicking Asp-68 in the first interface), Ser-72 (mimicking Gly-67), and Leu-76 (mimicking Leu-63) of the BaxBH3 domain and the hydrophobic groove of Bcl-x_L chain B. We believe that this second interface is an artifact of crystallization. As in the Mcl-1·BaxBH3 complex, the region of Bax visible (54–79) in the crystal is a continuous helix, unlike the equivalent region of the inactive

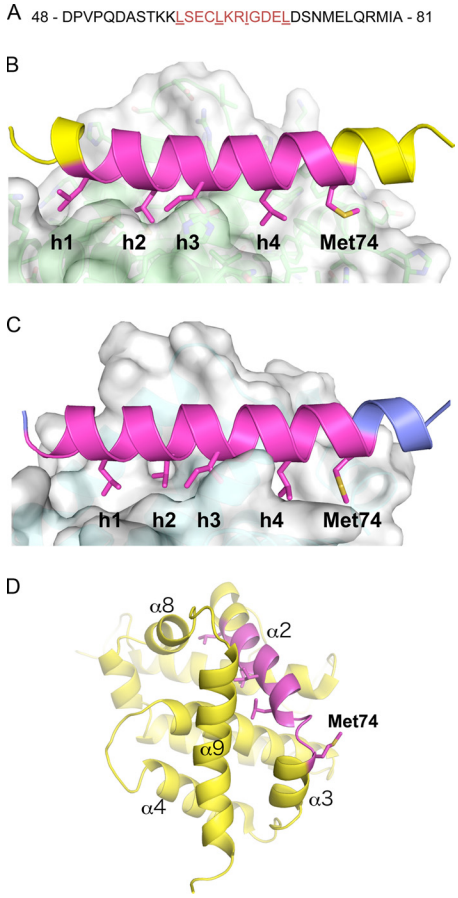

FIGURE 1. **Crystal structures for the BaxBH3 domain and flanking regions in complex with Mcl-1 and Bcl-x_L.** *A*, Bax peptide sequence used for crystallization. The canonical BH3 domain of Bax, as delineated by the conserved hydrophobic residues (*underlined*), is colored *red*. *B*, crystal structure of Mcl-1·BaxBH3. *C*, crystal structure of Bcl-x_L·BaxBH3. *D*, the structure of inactive, soluble Bax (8). In *B* and *C*, the peptide ligand is colored *yellow* and *blue*, respectively, with the region in contact with the pro-survival hydrophobic groove colored in *magenta*. The equivalent region in inactive Bax is also colored *magenta* in *D*. Note that Met-74 is buried and located on the same side as the four conserved hydrophobic BH3 domain residues when in complex with a pro-survival protein, but solvent-exposed and transverse to the hydrophobic residues in inactive Bax.

TABLE 1

Crystallographic statistics for structures of the Mcl-1·BaxBH3 complex and Bcl-x_L·BaxBH3 complex

	Mcl-1·BaxBH3	Bcl-x_L·BaxBH3
Data collection		
Space group	C2	$C222_1$
Cell dimensions *a*, *b*, *c* (Å)	97.9, 81.5, 57.9, $\beta = 124.2$	70.2, 99.0, 113.1
Wavelength (Å)	0.95364	0.95561
Resolution range (Å)	50.00–2.50 (2.59–2.50)	50.00–2.60 (2.69–2.60)
R_{merge}	0.105 (0.641)	0.105 (0.591)
$I/\sigma I$	41.5 (2.4)	26.4 (6.1)
Completeness (%)	98.6 (94.1)	99.9 (100.0)
Redundancy	5.8 (4.9)	14.6 (14.5)
Refinement		
Resolution (Å)	20.1-2.50 (2.74-2.50)	33.55-2.60 (2.87-2.60)
No. of reflections, R_{work}	12,394 (2959)	12,216 (2795)
No. of reflections, R_{free}	671 (152)	587 (143)
R_{work}	0.21 (0.31)	0.19 (0.23)
R_{free}	0.25 (0.36)	0.25 (0.33)
No. of atoms		
Protein	2673	2502
Water/ions	67	38
r.m.s. deviations[a]		
Bond lengths(Å)	0.008	0.008
Bond angles (°)	1.084	1.021
Chiral	0.065	0.068

[a] r.m.s., root mean square.

TABLE 2

Analysis of binding of Bax Met-74 mutant peptides to pro-survival proteins using a Biacore-based competition assay

Values are presented as an IC_{50} in nM, and values in brackets represents S.D. (n = 2–3 assays).

	Bcl-x_L	Bcl-w	Bcl-2	Mcl-1
WT BaxBH3	184 (30)	67 (9)	94 (12)	101 (40)
BaxBH3 M74A	653 (23)	240 (23)	320 (11)	327 (60)
BaxBH3 M74R	176 (41)	76 (16)	289 (40)	359 (75)
BaxBH3 M74K	210 (15)	70 (5)	665 (75)	425 (75)
BaxBH3 M74D	2575 (235)	690 (5)	3350 (470)	8400 (370)
BaxBH3 M74E	4150 (29)	1215 (115)	3750 (360)	3597 (690)

Bax structure, and Bax Met-74 binds to a fifth hydrophobic pocket within the peptide-binding groove of Bcl-x_L.

BaxBH3 Peptides Containing Met-74 Mutations Are Compromised in Their Binding to Pro-survival Bcl-2 Proteins—As noted, the orientation of Bax Met-74 with respect to h1–h4 of the BaxBH3 differs significantly in the Mcl-1·BaxBH3 and Bcl-x_L·BaxBH3 structures presented here as compared with the inactive Bax structure (Fig. 1). Solution binding studies were performed to investigate the significance of this residue in binding pro-survival proteins. IC_{50} values were calculated from competition binding experiments using surface plasmon resonance (Table 2). The values we obtained for the wild-type BaxBH3 sequence using this technique are consistent with previous reports using isothermal titration calorimetry (17). In the context of 34-mer peptides, M74A, M74R, and M74K mutations result in a loss of up to 6-fold binding affinity to those pro-survival proteins found in mouse embryonic fibroblasts (Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1), whereas the M74E and M74D mutations result in a 10–80-fold loss of binding to these proteins. These data support the conclusion from the structural studies that Bax Met-74 contributes to the binding energy of the peptide to the proteins.

Mutation at Bax Met-74 Disrupts Binding of Bax to Pro-survival Proteins in Mammalian Cells—We next sought to determine whether Met-74 participates in the interaction between full-length Bax and pro-survival Bcl-2 proteins in cells. FLAG-tagged wild-type Bax and Met-74 mutants were co-expressed in 293T cells with HA-tagged pro-survival proteins to investigate the role of this residue in interactions between full-length proteins in cells (Fig. 2). Immunoprecipitations were performed using anti-FLAG antibody to pull down Bax or Bax mutants, and blots were probed for pro-survival proteins using anti-HA antibody. Although the interpretation of immunoprecipitations can be complicated by indirect interactions, in these experiments, Bax Met-74 mutants are compromised in their binding to pro-survival proteins as compared with wild-type Bax, confirming the importance of the interaction observed in the crystal structure. For Bcl-x_L, Bcl-2, and Bcl-w, the pulldowns agree well with the peptide binding data. For Mcl-1, the agreement is less robust across the mutant series, possibly as a result of variations in Mcl-1 levels in these cells (for example, in Fig. 2, *panel 3*, *left column*, compare *lanes 4* and *5*), resulting from the tight regulation of this protein in cells by multiple mechanisms. Nevertheless, even for Mcl-1, in all cases, mutation at Met-74 compromises binding.

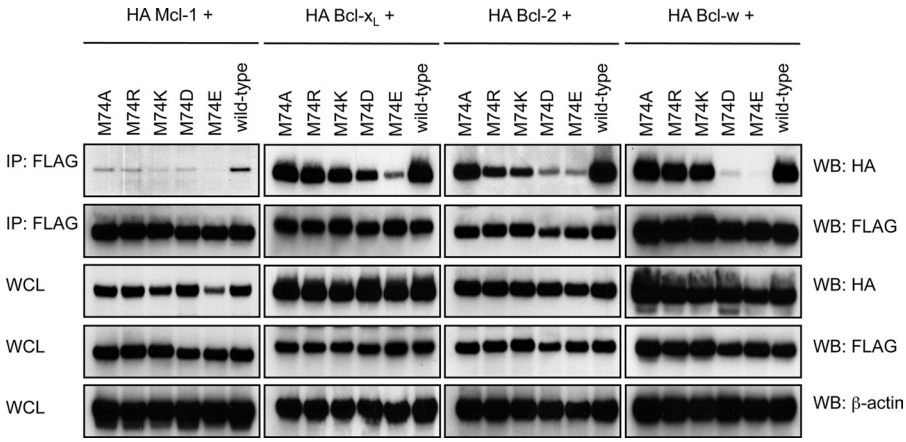

FIGURE 2. **Mutation of Met-74 disrupts binding of Bax to full-length pro-survival proteins in cells.** 293T cells were transfected with HA-tagged pro-survival proteins and FLAG-tagged Bax Met-74 mutants. Immunoprecipitations (*IP*) were performed using anti-FLAG antibody to pull down Bax, and Western blots (*WB*) were probed for pro-survival proteins using anti-HA antibody. Whole cell lysates (*WCL*) were probed with either anti-HA or anti-FLAG to confirm levels of protein expression.

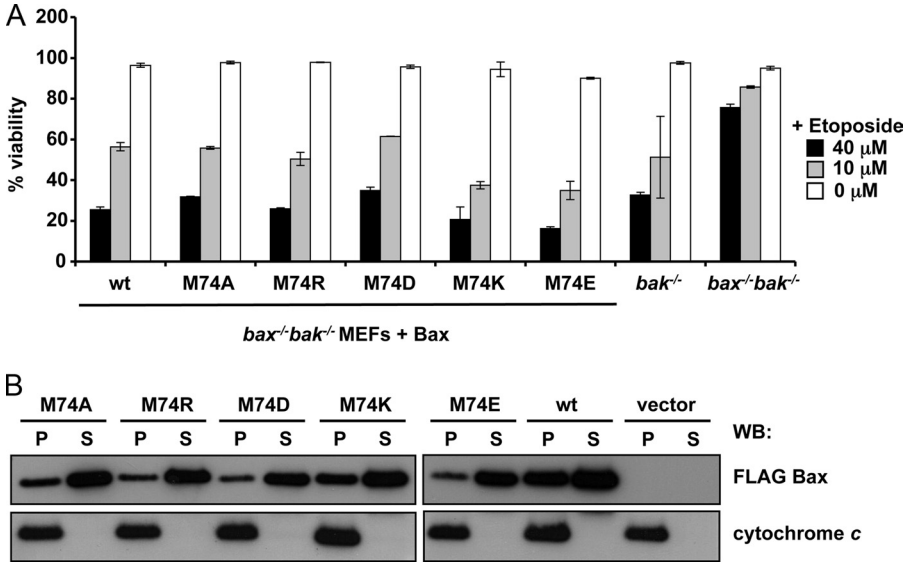

FIGURE 3. *A*, Bax Met-74 mutants are as effective as wild type in mediating cell killing in response to etoposide. Bax Met-74 mutants were expressed in $bax^{-/-}/bak^{-/-}$ MEFs and treated with etoposide or an equivalent volume of DMSO. Cell viability was monitored by propidium iodide exclusion. *Error bars* indicate S.D. *B*, Bax Met-74 mutants do not show enhanced association with mitochondria. The soluble (*S*) and pellet (*P*) fractions from permeabilized $bax^{-/-}/bak^{-/-}$ MEFs stably expressing FLAG-tagged Bax mutants were probed by Western blot (*WB*) for either FLAG-Bax or cytochrome *c*.

Mutation at Bax Met-74 Prevents Pro-survival Proteins from Rescuing Bax Growth Suppression in Yeast—It has previously been shown that expression of Bax in yeast inhibits growth and that co-expression with a pro-survival protein rescues this phenotype (40). Bax Met-74 mutants retain the ability to suppress yeast growth (supplemental Fig. 1), suggesting that they are functional. However, the ability of Bcl-2, Bcl-x$_L$, and Mcl-1 to rescue growth suppression in these assays is correlated with their ability to bind Bax Met-74 peptide mutants (Table 2). Bax M74E and M74D had the most significant effect, abolishing the ability of Bcl-w, Bcl-2, and Mcl-1 to inhibit Bax activity in yeast and severely reducing that of Bcl-x$_L$.

Bax Met-74 Mutants Retain Killing Activity in Mammalian Cells—To further characterize their capacity to mediate apoptosis, Bax Met-74 mutants were expressed in $bax^{-/-}bak^{-/-}$ MEFs. No significant difference was observed in the ability of wild-type Bax *versus* Met-74 mutants to mediate cell killing in response to the DNA-damaging drug, etoposide (Fig. 3*A*), As etoposide causes up-regulation of Puma, Bim, and Noxa (41–43), all pro-survival proteins within the cell are likely to be neutralized (44). This killing result demonstrates that all of the Bax mutants are able to translocate to the mitochondria, form oligomers, and permeabilize the outer mitochondrial membrane. Although this assay is only semiquantitative, it is noted that Bax M74D displayed a slight reduction in killing activity in response to etoposide (35% cell survival at 40 μM), whereas M74E demonstrated a slight increase in killing activity (17%) as compared with WT (25%). This raises the possibility that M74D has a reduced killing capacity and that M74E has an enhanced killing capacity in response to etoposide (but not in clonal viability assays; see below).

Bax mutants that preferentially localize to mitochondria (*e.g.* S184L (45)) have increased killing activity, so we determined the subcellular distribution of the Met-74 mutants in cells (in the absence of an external apoptotic stimulus)(Fig. 3*B*). In contrast to Bax S184L, all Met-74 mutants show decreased mem-

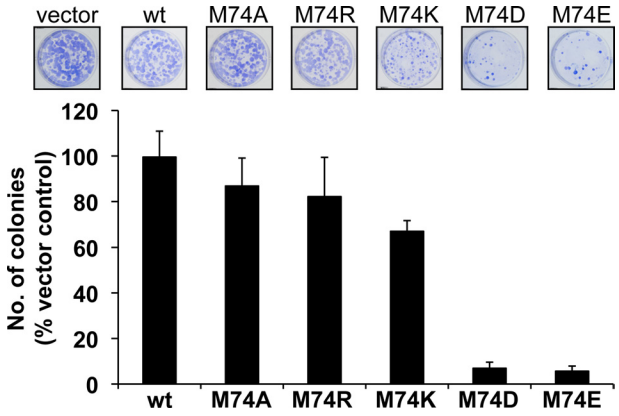

FIGURE 4. **Colony assays on *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing wild-type Bax and Met-74 mutants.** Cells were sorted directly into trays and colonies scored after 6 days. Expression of Bax M74E and M74D markedly suppressed colony formation. *Error bars* indicate S.D.

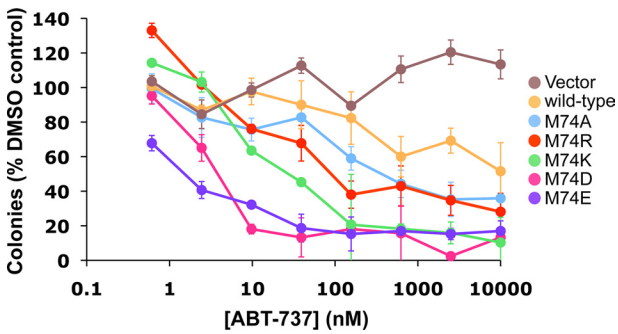

FIGURE 5. **Mutations to Bax Met-74 that disrupt binding to pro-survival proteins sensitize cells to ABT-737.** Colony assays were performed as in Fig. 4 with the exception that either ABT-737 or an equivalent DMSO control was added to the culture medium. *Error bars* indicate S.D.

brane localization as compared with wild type. Binding of Bax to pro-survival proteins (notably Bcl-x_L) on membranes is well established (21, 23), so this change in localization could relate to the observed reduction in the association of Bax Met-74 mutants with pro-survival proteins. In any event, any change in the pro-apoptotic activity of the mutants is not due to a more favorable (*i.e.* increased membrane) localization.

Cells Expressing Bax M74E and M74D Have Markedly Reduced Viability in Colony Assays—The MEF reconstitution experiments above were performed in cells sorted for GFP expression linked to Bax expression through an IRES sequence. We also attempted to generate MEF cell lines stably expressing these Bax mutants to further study their function. Cells were infected with retroviruses in which expression of Bax (or mutants) was linked to a hygromycin resistance cassette via an IRES sequence. Cell lines could be readily generated with wild-type Bax, Bax M74A, and Bax M74R. However, cells transfected with Bax M74E were 100% dead following 1 week under hygromycin selection. We questioned whether this mutant might be constitutively active as a result of reduced or abolished binding to the pro-survival Bcl-2 proteins present in these cells. Alternatively, cells expressing this mutant could be abnormally sensitive to apoptotic stimuli encountered during normal culturing. In this scenario, apoptosis ensues due to the reduced capacity to restrain Bax M74E once activated through stimuli arising from the stresses of cell culture. To quantitate the level of cell death observed, we performed colony assays on *bax*$^{-/-}$*bak*$^{-/-}$ MEFs expressing Bax Met-74 mutants. As with previous experiments, Bax expression was linked to GFP expression through an IRES sequence. Cells that expressed GFP were sorted into trays, and colonies were scored after 6 days. The level of colony formation (Fig. 4) correlates with the loss of binding of Bax Met-74 mutant peptides to pro-survival proteins, the Ala, Lys, and Arg mutants displaying a weaker phenotype than the Asp and Glu mutants.

Bax Met-74 Mutants Enhance Susceptibility to Killing by ABT-737—ABT-737 is a *bona fide* antagonist of Bcl-2, Bcl-x_L, and Bcl-w, but not Mcl-1 (46), and as a result, resistance is correlated with Mcl-1 levels (47, 48). As the various Bax Met-74 mutants possessed reduced affinity for Mcl-1 as compared with wild-type Bax, we suspected that their expression in cells might result in increased sensitivity to ABT-737 as compared with those expressing the wild-type protein if the role of the pro-survival is to inhibit Bax directly. Hence, we repeated the colony assays described above, this time adding ABT-737 to the cultures. The colonies were scored as a percentage of the DMSO control (Fig. 5). As expected, the *bax/bak* double knock-out cells do not undergo apoptosis, and cells reconstituted with wild-type Bax show only modest killing at very high concentrations of ABT-737 due to the presence of Mcl-1 in these cells. In contrast, cells transfected with the mutants are increasingly sensitive to ABT-737 through the series M74A, M74R, M74K, M74D, and M74E. Generally, the stronger the interaction between the mutant Bax peptide and Mcl-1, the less sensitive are the cells to killing by ABT-737. These data illustrate that the protective effect of Mcl-1 against ABT-737 can be mediated by interactions between Bax and Mcl-1 and not necessarily by Mcl-1 sequestering BH3-only proteins.

DISCUSSION

We describe here structures for peptides encompassing the BaxBH3 domain in complex with the pro-survival proteins Mcl-1 and Bcl-x_L. These structures are similar to previously solved complexes of pro-survival proteins with BH3 domains from BH3-only proteins. In the new structures reported here, the BaxBH3 domain and the sequence C-terminal to it form an amphipathic helix that extends along the length of the hydrophobic groove of both Mcl-1 and Bcl-x_L. In contrast, the secondary structure of the equivalent region of inactive Bax comprises part of helix α2 and the turn linking α2 and α3 (Fig. 1). Significant structural rearrangements are therefore required of inactive Bax to form the interface observed in our complexes of Mcl-1·BaxBH3 and Bcl-x_L·BaxBH3. Not only must α2 become dislodged from the helical bundle to reveal its buried hydrophobic surface, but the adjoining N terminus of α3 must also become continuous with the everted α2. Reduced interactions in cells between Bax Met-74 mutants and pro-survival proteins could be due either to weakening the complex as visualized in the structures of BaxBH3 bound to Mcl-1 and Bcl-x_L or to the imposition in the mutants of an energy barrier to eversion of the BaxBH3. The latter explanation is at odds with the observation that the mutants are

fully (indeed excessively) active and are able to undergo whatever conformational changes such activity requires. Therefore we conclude that Met-74 is an important residue in the formation of complexes between full-length Bax and pro-survival proteins in cells.

Most BH3-only proteins are unstructured in solution, yet their BH3 domains adopt a helical structure upon binding to pro-survival proteins (6). An exception is Bid, which adopts a similar fold to the multidomain Bcl-2 proteins in which the hydrophobic face of the BH3 domain is also buried (49, 50). Protease cleavage is required for activation of Bid, presumably relaxing the structure to allow exposure of its BH3 domain. In contrast, inactive Bak and Bax adopt distinct folds in solution that resemble those of pro-survival Bcl-2 family proteins (7, 8). Like Bid, the hydrophobic faces of the BaxBH3 and BakBH3 domains are buried in these inactive structures. Activation of Bax and Bak results in conformational changes as detected by antibodies recognizing buried epitopes in the inactive structure (45, 51, 52). During, or perhaps as a consequence of, this structural remodeling, Bax forms into large homo-oligomeric complexes on the outer mitochondrial membrane. This results in the release of cytochrome *c* from the mitochondria by an unknown mechanism. Our observation that mutation to Bax Met-74 disrupts interactions with full-length pro-survival proteins in cells and inhibits the ability of pro-survival proteins to rescue Bax activity in yeast cells suggests that the orientation of this residue as found in our structures is representative of full-length complexes in cells. Bax Met-74 is located not within, but adjacent to, the canonical BH3 domain of Bax. The observation that exposure of Bax α2 is accompanied by it becoming collinear with the first turn of α3 raises the possibility that other residues beyond the BaxBH3 domain may also mediate interactions with pro-survival proteins. However, as Bax Met-74 mutants retain killing activity, this suggests that this residue is not critical for Bax homo-oligomerization, which, based on current models for Bak oligomerization (53, 54), also likely involves a BH3-in-groove interaction.

Unlike the Bax S184L mutation (45), which drives Bax to the mitochondrial membrane, increasing its toxicity, Bax Met-74 mutants display somewhat reduced mitochondrial localization as compared with wild-type Bax. This may be due to weakened interactions with pro-survival Bcl-2 family proteins at the mitochondrial membrane. Indeed, recent studies have shown that Bax at the mitochondrial membrane can interact with both direct activators and membrane-bound pro-survival proteins (21, 23). Whatever the reason for the enhanced cytosolic distribution of Bax Met-74 mutants, their enhanced killing activity in the absence of an apoptotic stimulus must relate to some property of the mutants after they have localized to mitochondria. We propose that property to be weakened interactions with pro-survival proteins.

Met-74 is on the opposing face of Bax to the putative binding surface for "activator" BH3-only proteins (16) (supplemental Fig. 2). Furthermore this residue does not project into the BaxBH3-binding groove, which has also been proposed as a binding site for activator BH3-only proteins (4). These structural considerations make it unlikely that expression of proteins bearing this mutation sensitize cells to apoptosis through enhanced sensitivity to direct Bax activation by BH3-only proteins. Instead the toxicity of these mutants when expressed in cells correlates with the ability of pro-survival Bcl-2 family proteins to bind to and inhibit the Bax variants, even in the absence of a death signal. Experimental confirmation of this position is frustrated by our failure to date to express Bax Met-74 mutants in sufficient quantity for biochemical experiments.

As Bax Met-74 lies adjacent to, rather than within, the BaxBH3 domain, it has been largely ignored in previous mutagenesis studies. An exception is the work by Wang *et al.* (24) that predates determination of the Bax solution structure (8) and incorrectly assumed that Met-74 would lie on the same hydrophobic face as h1–h4 in inactive Bax. They found that Bax M74A disrupted interactions with Bcl-2 and Bcl-x_L in a yeast two-hybrid system but did not significantly inhibit the pull down of full-length proteins in cells. In addition, Bax M74A did not inhibit homo-oligomerization, consistent with our observations. Although expression of this mutant was found to induce apoptosis in Jurkat cells, control killing experiments were not performed as the work predates the development of $Bax^{-/-}Bak^{-/-}$ cell lines (55, 56).

While this manuscript was under review, a study of murine Bax regulation by Bcl-2, including a crystal structure for a BaxBH3 36-mer peptide bound to Bcl-2, was published (PDB entry 2XAO) (57). Those authors focused on ionic interactions between the peptide and the protein, and they characterized a triple mutant (E61A/R64A/R78A) that can no longer be restrained by Bcl-2 but remains responsive to etoposide treatment. Whether or not this mutant is free from other pro-survival protein restraint is not addressed, nor is the viability of cells expressing the mutant analyzed in longer term colony assays. However, for crystal contacts, homologous ionic interactions might also have been observed in the Bcl-x_L complex reported here. Met-74 of the BaxBH3 peptide makes a similar interaction with Bcl-2 as it does with Bcl-x_L and Mcl-1, and the M74A mutant peptide has reduced (14×) binding to Bcl-2 as measured by isothermal titration calorimetry. These results are in general accord with our study, although the Met-74 mutant is not characterized in full-length Bax. In contrast with our data and other published work (17), pulldowns fail to show an interaction with Bax and either Bcl-x_L or Mcl-1, but no cellular experiments directly address whether these proteins control Bax (57).

Pro-survival Bcl-2 family proteins are currently being pursued in a number of cancer therapeutic discovery projects (46, 58). Understanding the primary role of pro-survival proteins in inhibiting apoptosis is important not only for future compound development but also for understanding potential resistance mechanisms. It is now well understood that a common resistance phenotype of cells to ABT-737 is the presence of the pro-survival protein Mcl-1, which is not antagonized by ABT-737. Whether this resistance is mediated by Mcl-1 restraining pro-apoptotic Bax and Bak or by

Mcl-1 sequestering pro-apoptotic BH3-only proteins has been contentious. Our data demonstrate that one model for resistance to ABT-737 depends on Mcl-1 restraint of Bax, and likely also of Bak.

Acknowledgments—Crystallization trials were performed at the Bio21 C3 Collaborative Crystallisation Centre. Data were collected at the Australian Synchrotron, Victoria, Australia, and we thank staff from the macromolecular beamlines. We thank Abbott Laboratories for provision of ABT-737 and David Huang and Jerry Adams for helpful discussions and critical comments on the manuscript.

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